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Activity of gut proteinases from Cry1Ab-selected colonies of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae)

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Abstract: Susceptibility to the Cry1Ab protoxin and toxin from *Bacillus thuringiensis* (Berliner) and activity of gut proteinases were assessed in both susceptible and Cry1Ab-selected colonies of European corn borer, *Ostrinia nubilalis* (Hübner). Resistance in two different selected colonies was at least 6- and 15-fold for the Cry1Ab protoxin and 108- and 484-fold for the Cry1Ab toxin. Activities of trypsin-like, chymotrypsin-like and elastase-like proteinases were variable among the colonies tested and not indicative of a major contribution to Cry1Ab resistance. Activation of the 130-kDa Cry1Ab protoxin occurred rapidly in all colonies, with no apparent differences among colonies. In addition, there were no apparent changes in activated Cry1Ab processing, indicating that proteolytic degradation was not associated with resistance. These results suggest that mechanisms other than proteolytic activation of protoxin and toxin degradation, such as target site modification may be involved in the resistance to *B. thuringiensis* Cry1Ab in these *O. nubilalis* colonies.

Keywords: transgenic corn, proteolytic enzymes, *Bacillus thuringiensis,* resistance, serine proteases

1 INTRODUCTION

Pest resistance evolution among insect pest species is a major concern related to the use of *Bacillus thuringiensis* (Bt) (Berliner) toxins in transgenic plants. Bt-based products have been used for more than 20 years without reported resistance, perhaps due to low selection pressure exerted by early formulations and usage patterns.[1] However, the first case of resistance was reported for *Plodia interpunctella* (Hübner) in 1985,[2] which increased concern that pests could evolve resistance to Bt products. Subsequently, many other reports of Bt resistance in pests studied under laboratory conditions have been documented (for reviews see References [3-6]); however, the only example of field resistance was reported for the diamondback moth, *Plutella xylostella* (L) after repeated and prolonged use of a Bt formulation.[4,5,7]

Understanding how insect pests develop Bt resistance is important to the concept of resistance management. To date, the best-characterized mechanism of Bt resistance in insect pest species involves altered binding to specific receptors in the insect midgut,[5] although other mechanisms have been suggested given the multiple steps involved in Bt mode of action. One such mechanism involves reduced toxin activation in the midgut, which has been associated with resistance in several species.[8-11] After solubilization under alkaline conditions (>pH 10), Bt toxins are activated by gut proteinases and then bind to specific receptors in the microvilli of cell membranes. Specific receptor binding is believed to elicit pore formation that alters the osmotic balance in the gut, leading to cell lysis and rupture of the gut lining.[4,12] Changes in the activity of the gut proteinases responsible for toxin activation could lead to reduced susceptibility to Bt toxins.

Bt toxins undergo hydrolysis by digestive enzymes referred to as proteinases. These enzymes are endopeptidases that act on peptide bonds, and, together with the exopeptidases, make up the peptidases or proteases.[13] Many of the reported Bt resistance studies involving endopeptidases included

serine proteinases such as trypsin-like and chymotrypsin-like proteinases, which are involved in the mechanism of Bt resistance in several insect species.[8,11,14,15] In *P. interpunctella*, resistance to the Cry1Ab protoxin was 10-fold higher than to the activated toxin,[16] and the activity of serine proteinases was significantly reduced in the resistant insects. Although these results suggest the involvement of reduced proteinase activity in the resistance mechanism, the basis of suppressed activity was not identified.[14]

The European corn borer, *Ostrinia nubilalis* (Hübner), is an important pest of corn in the USA.[17] Although Bt products have been used to control *O. nubilalis* for at least 40 years, there are no reported instances of resistance. However, the risk of resistance evolution in *O. nubilalis* to Bt toxins expressed by transgenic corn is considered high because of elevated expression levels in the plant and because insects are exposed throughout larval development. The potential for resistance development has been demonstrated in laboratory-selected colonies of *O. nubilalis* exposed to Dipel-ES,[18] Cry1Ac[19] and Cry1Ab toxin.[20] Selection with Dipel-ES resulted in 73-fold higher LC_{50} values after seven generations of selection,[17] and selection with a Bt formulation (MVP) that contained only Cry1Ac resulted in resistance levels up to 162-fold after 17 generations, although resistance levels declined rapidly after selection pressure was removed. Cry1Ab-selected colonies showed variable levels of resistance, with a peak of 14-fold after seven generations of selection.[20] Characterization of Bt resistance mechanisms in the Dipel-selected colonies of *O. nubilalis* reported by Huang *et al*[11] suggested that the activities of serine proteinases such as chymotrypsin-like and trypsin-like proteinases were reduced relative to susceptible strains, indicating involvement of reduced rates of toxin activation.[14,21,22]

Proteolytic activation of Cry toxins is critical not only for protoxin activation but also has implications for toxin specificity,[22,23] receptor binding[24] and insect resistance.[11,15] Because little information exists on the mechanisms of Bt resistance among *O. nubilalis* populations, the objective of the present work was to examine the involvement of proteinases from Cry1Ab-selected colonies previously shown to have developed significant levels of resistance to both protoxin and bovine trypsin-activated Cry1Ab.[20,25] Two resistant colonies and a standard susceptible control were assayed for proteinase activity in luminal gut contents to determine their potential involvement in Cry1Ab resistance.

2 EXPERIMENTAL

2.1 Insect rearing

Rearing procedures for *O. nubilalis* were based on those developed at the USDA-ARS Corn Insects Research Unit, Ames, IA, by Guthrie *et al*.[26] Larvae were reared at 27 (±0.7) °C and 80% RH under a 24:0 h light:dark photoperiod on a wheat germ-based diet.[27] Insects were moved to mating cages as pupae where adults were maintained with 8-h scotophase at 18 (±0.7) °C and 16-h photophase at 27 (±0.7) °C with 80% RH. Cages were misted with water twice daily, and adult diet was provided to maximize egg production.[28] Egg masses were collected and incubated within plastic Petri dishes containing moistened filter paper until larval hatch.

2.2 *Ostrinia nubilalis* colonies

Three populations of *O. nubilalis* designated Europe-S, Europe-R and RSTT-R were established in the laboratory. The European colony was established in 1993 from approximately 500 *O. nubilalis* larvae collected in the Lombardia region of northern Italy and was provided to the University of Nebraska after 20 generations of laboratory rearing. This colony was divided into two sub-populations: one exposed throughout development to Cry1Ab protoxin from the *B. thuringiensis kurstaki* strain HD1-9, which produces only Cry1Ab protein[29] (Europe-R), and the other reared in the absence of protoxin (Europe-S). The RSTT-R colony resulted from a combination of individuals from both a Nebraska- and Europe-selected colonies. All the resistant colonies were selected with full-length Cry1Ab toxin for at least 40 generations.

2.3 *Bacillus thuringiensis* Cry1Ab toxins

Purified Cry1Ab protoxin was obtained from the *B. thuringiensis kurstaki* HD1-9 strain, which produces only the Cry1Ab protein. This material was provided by Syngenta Seeds (Research Triangle Park, NC). The crystal protein was prepared by density gradient centrifugation and contained approximately 98% crystal protein as determined by phase contrast microscopy. The activated Cry1Ab was obtained from the Monsanto Company (St Louis, MO). Fermentation slurry containing the Cry1Ab protoxin was treated with bovine pancreatic trypsin at 4 °C until approximately 90% was converted to the trypsin-resistant core protein. After dilution of the supernatant, the protein was loaded onto a 1.2-liter column of Q Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ). The trypsin-resistant core protein was eluted as a single symmetrical peak by using a sodium chloride gradient. Peak tubes were pooled and dialyzed against carbonate/bicarbonate buffer (50 mM, pH 10.25) with sodium chloride (50 mM). The trypsin-resistant core protein was recovered at 94% purity.

2.4 Bioassays

Bioassays were conducted with both full-length and activated Cry1Ab. The method described by Marçon *et al*[30] was used for both control and selected strains and involved exposure of neonates (<24 h after hatching) to purified Bt toxins evenly overlaid onto the surface of an artificial diet. The diet devel-

oped for *Heliothis virescens* (F)[31] was used in place of rearing diet for all bioassays. One milliliter of diet was dispensed into each well of 128-well trays (each well 16 mm in diameter, 16 mm in height; CD international, Pitman, NJ) and allowed to solidify. Seven concentrations of each toxin were tested, and dilutions were prepared in 1 ml liter^{-1} Triton X-100 non-ionic detergent to obtain uniform spreading onto the diet surface. Each well was treated with 30 µl of the appropriate solution and allowed to air dry for 1 h. Controls were treated with 1 ml liter^{-1} Triton X-100 only. A single neonate (<24 h after hatching) was placed into each well, and the wells were then covered with vented lids (CD International). Trays were held for 7 days at 27 °C, 24-h scotophase and 80% RH. Mortality was recorded after 7 days. Larvae that did not grow beyond first instar were considered dead. Thus, the criterion for mortality used in this study accounted for both severe growth inhibition and death. Each bioassay was replicated at least three times for each population. Probit analysis[32] was conducted using the POLO-PC package.[33] Likelihood ratio tests for parallelism and resistance ratio (ie LC_{50} ratios) comparisons were performed according to Robertson and Preisler.[34] Resistance ratios were considered significantly different ($P < 0.05$) when the confidence limits did not include the value 1.

2.5 Insect gut extraction

Gut proteinases were obtained from *O. nubilalis* late fifth instars using the method described by Oppert *et al*[14] with slight modification. Selected colonies were reared in the presence of Cry1Ab protoxin before gut contents extraction. Larvae were chilled, and the midguts were pulled from the carcass after excision of the head and thorax and last three abdominal segments. Guts were removed with forceps and immediately submersed in ice-cold buffer A (Tris 200 mM, pH 9.0, calcium chloride 20 mM). Five guts were aliquoted per 100 µl of buffer A, and at least 10 samples of each colony were frozen at -20 °C until used in subsequent activity assays. Samples were quick-thawed by spinning at 15 000*g* for 3 min, and the supernatants containing soluble gut proteinases were filtered using a 0.22-µm microfilter (Millipore Corporation, Bedford, MA). Protein concentrations were determined by the bicinchoninic acid method[35] with bovine serum albumin as a standard. The protein concentration of each preparation was standardized to 6 mg total protein ml^{-1}.

2.6 Total protease activity

Total activity of luminal proteases was measured using azocasein as substrate by modification of the method described by Loseva *et al.*[36] Gut extracts were diluted to 2 mg protein ml^{-1} and 50 µl was incubated with buffer A (450 µl) containing sodium chloride (0.15 M) and Tris-HCl (20 mM, pH 9.0) for 20 min at 37 °C before addition of azocasein solution in the same buffer (5.0 g liter^{-1}, 500 µl). The reaction was allowed to proceed for 1 h at 37 °C before it was stopped with trichloroacetic acid solution (100 g liter^{-1}, 500 µl). Solutions were centrifuged at 16 000*g*, and absorbance of the supernatant was measured at 440 nm. Control reactions were identical except for the absence of gut extracts. The rate of proteolysis of azocasein was expressed in mOD_{440} (milli optical density at 440 nm) per minute per milligram of gut content protein. Analysis of variance was performed to determine treatment effects using PROC analysis of variance (ANOVA),[37] and means were separated by Fisher's least significant difference (LSD) test at $P < 0.05$.

2.7 Specific proteinase assays

Synthetic proteinases substrates, *N*-α-benzoyl-L-arginine *p*-nitroanilide (Ba*p*NA), *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide (SAAPF*p*NA) and *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide (SAAPL*p*NA), were purchased from Sigma-Aldrich (St Louis, MO) and used to determine the trypsin-like, chymotrypsin-like and elastase-like proteinase activities, respectively. Enzyme activities were determined at 30 °C by using a kinetic microplate reader (Biotek, Winooski, VT) at 405 nm. Kinetic analysis was performed with 10 concentrations, ranging from 1.0 µg to 1.0 mg substrate ml^{-1} (ie 2.3 µM to 2.3 mM BA*p*NA, 1.6 µM to 1.6 mM SAAPF*p*NA, and 1.7 µM to 1.7 mM SAAPL*p*NA), dissolved in buffer A at 30 °C. Proteinase extract (100 µl of 100-fold diluted proteinase extract: 6 µg of total protein) was mixed with 100 µl of substrate solution in a 96-well microplate. Changes in absorbance at 405 nm were recorded for 5 min at 11-s intervals. Initial velocities were used for kinetic analysis of all three substrates. Proteinase activities for all three substrates were calculated based on the molar extinction coefficient of 8800 for the reaction product *p*-nitroaniline. Michaelis constant (K_m) and maximal velocity (V_{max}) estimates for each substrate were determined by non-linear regression based on the Michaelis-Menten model.[38] ANOVA was applied to compare K_m and V_{max} among different *O. nubilalis* strains for each substrate. A Fisher's LSD test was used for mean separation.

2.8 Luminal activity toward Cry1Ab *in vitro*

To determine whether the differences in proteolytic activity correlated with differences in Cry1Ab protoxin activation, peptides resulting from Cry1Ab incubations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Trypsinized Cry1Ab also was analyzed to determine whether proteolytic degradation of toxin was involved in the resistance. Purified Cry1Ab protoxin and truncated Cry1Ab were dissolved in sodium carbonate/sodium hydrogen carbonate buffer (50 mM, pH 10.0), at a concentration of 2 mg ml^{-1}, equilibrated at 30 °C, and mixed with gut content extracts (30 °C) in a protoxin:gut extract ratio of 3:1 by volume. Reaction mixes were incubated for 30 and 60 min and 24 h at 30 °C. Immediately after the incubation, samples were diluted 2:1 with Laemmli buffer and incubated at 95 °C for 5 min to denature the proteins. Both Cry1Ab protoxin and toxin

hydrolysis by *O. nubilalis* midgut extracts were compared by SDS-PAGE. Precast Criterion gels (10%, Tris-HCl) were purchased from Bio-Rad (Hercules, CA). Gels were stained with Coomassie Brilliant Blue R-250 (1.0 g liter^{-1}) in methanol + acetic acid (400 + 100 g liter^{-1}) solution.

3 RESULTS

3.1 Bioassays

Two independent Bt-resistant colonies of *O. nubilalis* were obtained by selection against Cry1Ab protoxin over 40 generations. The toxicities of both Cry1Ab protoxin and toxin (produced by treatment with bovine trypsin) to susceptible corn borer larvae (Europe-S), and two resistant-selected laboratory colonies (Europe-R and RSTT-R), are presented in Table 1. The RSTT-R colony exhibited consistently higher levels of resistance at the LC_{50} to both the protoxin (15-fold) and trypsinized toxin (484-fold) than did the Europe-R colony (6- and 108-fold, respectively). Both the Europe-R- and RSTT-R-selected colonies were more tolerant (18- and 32-fold) to trypsin-activated Cry1Ab than to Cry1Ab protoxin. In contrast, the Europe-S colony was approximately 3-fold less tolerant to trypsinized Cry1Ab than to Cry1Ab protoxin (Table 1).

3.2 Total proteinase activities

Luminal gut extracts from the resistant Europe-R colony exhibited reduced hydrolysis of azocasein (16%) compared with the susceptible colony, whereas activity of the RSTT-R colony showed slightly higher (4.6%) activity toward this substrate (Fig 1). Although total proteinase activities among all three colonies were statistically different ($P < 0.05$), there was no apparent correlation with the levels of resistance.

3.3 Specific proteinase activities

The luminal gut contents were assayed for three specific proteinases over a range of 10 substrate concentrations. The data for the resistant and susceptible colonies of *O. nubilalis* are presented in Fig 2. When compared with the susceptible Europe-S colony, the two selected colonies exhibited reduced activity of both elastase-like and chymotrypsin-like proteinases.

Table 1. Toxicity of Cry1Ab protoxin and toxin towards a Cry1Ab-susceptible and two Cry1Ab-resistant colonies of *Ostrinia nubilalis*[a]

Colony	LC_{50} (95% FL)[b] (ng cm^{-2})	LC_{95} (95% FL)[b] (ng cm^{-2})	Slope[c]	RR (95% FL)[de]	χ^{2f}
		Cry1Ab protoxin			
Europe-S	18.3 (13.3–33.1)	123.0 (56.6–652.8)	1.99 (±0.38) a	—	3.70
Europe-R	104.1 (78.4–133.4)	478.1 (339.1–805.3)	2.49 (±0.32) a	5.7 (3.48–9.27)	0.95
RSTT-R	277.3 (144.9–521.9)	2714 (1205–13 827)	1.66 (±0.18) a	15.2 (8.91–25.68)	9.71
		Trypsinized Cry1Ab toxin			
Europe-S	6.50 (4.32–9.19)	33.8 (20.6–88.8)	2.30 (±0.32) a	—	5.48
Europe-R	700.7 (506.3–939.4)	6,057 (3994–10 887)	1.76 (±0.19) ab	107.8 (72.12–161.26)	2.94
RSTT-R	3144 (1152–6629)	41 552 (16 240–4.0 × 10^5)	1.47 (±0.17) b	483.7 (310.52–754.04)	13.37

[a] Data are based on three replications at each dose (seven doses in the total), and each bioassay comprised 336 larvae tested.
[b] Nanograms of Bt toxin per square centimeter of treated artificial diet surface.
[c] Values followed by the same letter within a column are not significantly different ($P \geq 0.05$). Significance of differences among slopes determined by likelihood ratio test of equality followed by pairwise comparisons by using non-overlapping fiducial limits.[44]
[d] Resistance ratio; ratio of the LC_{50} values between selected colonies and the nonselected colony, calculated using the method of Robertson and Preisler.[34]
[e] Fiducial limits (95%) for the lethal concentration ratio. If the interval contained the value 1.0, the LC_{50} values were not significantly different.
[f] Chi square significant ($P < 0.05$).

Table 2. Kinetics parameters of luminal proteinases from a Cry1Ab-susceptible and two Cry1Ab-resistant colonies of *Ostrinia nubilalis*[a]

Substrate	Colony	V_{max} (mmol min^{-1} mg^{-1}) (±SEM)	K_m (mM) (±SEM)	Activity relative to Europe-S (%)
	Europe-S	0.39 (±0.04) b	0.18 (±0.01) b	100.0
BA*p*NA	Europe — R	0.25 (±0.02) c	0.16 (±0.01) b	64.1
(Trypsin)	RSTT-R	0.87 (±0.05) a	0.41 (±0.05) a	223.1
	Europe-S	0.62 (±0.08) a	2.01 (±0.16) a	100.0
SAAPP*p*NA	Europe — R	0.30 (±0.04) b	1.59 (±0.09) b	48.4
(Chymotrypsin)	RSTT-R	0.36 (±0.04) b	1.66 (±0.04) b	58.1
	Europe-S	0.045 (±0.003) a	0.324 (±0.013) a	100.0
SAAPL*p*NA	Europe-R	0.025 (±0.005) b	0.358 (±0.022) a	55.6
(Elastase)	RSTT-R	0.026 (±0.001) b	0.265 (±0.022) b	57.8

[a] Data are the mean of nine assays ($n = 9$), each with three determinations. Regressions were significant at $P < 0.001$. Means within columns, for each substrate, with the same letter are not significantly different ($P < 0.05$, LSD test).

However, trypsin-like activity was significantly higher in the RSTT-R colony and significantly lower in the Europe-R colony relative to the unselected colony.

Significant differences were observed among the colonies for both the K_m and V_{max} values of these three enzyme activities (Table 2). The hydrolysis efficiency, measured by V_{max}, was significantly different among the colonies for each of the substrates tested (Table 2). Except for the higher rate of hydrolysis for trypsin-like activity in the RSTT-R colony (>2-fold), all the hydrolysis efficiencies were lower for the two resistant colonies when compared with those from the unselected Europe-S colony. The reduction in hydrolysis ranged from 36 to 52% for a given substrate (Table 2). In addition, the RSTT colony exhibited K_m values for all enzyme activities that were significantly different from the susceptible Europe-S colony with a comparatively higher K_m value for trypsin activity. The K_m value for chymotrypsin-like activity of the Europe-R was the only value significantly different from that of susceptible colony. In both resistant colonies chymotrypsin values of K_m were significantly lower than the value from the susceptible colony (Table 2).

3.4 *In vitro* Cry1Ab protoxin and Cry1Ab processing

Both selected and control colonies were able to completely hydrolyze the full-length Cry1Ab (protoxin) within 30 min (Fig 3, lanes 4-9). Although two major bands were observed within a range of 60-70 kDa (Fig 3, lanes 4-9), only one of them corresponded to the position of the activated toxin at 65 kDa (Fig 3, lanes 10-15). Additional incubation up to 24 h completed the proteolytic activation of protoxin to a single band corresponding to that of the bovine trypsin activated toxin (Fig 4, lanes 4-6), but there were no apparent differences in the patterns of the three insect colonies. Incubation of the bovine trypsin activated toxin with gut proteinases (Fig 4, lanes 7-9) showed some lower molecular mass degradation products, indicating that some of the protein toxin was degraded over time, although there were no differences in the rate of degradation among the resistant and susceptible colonies.

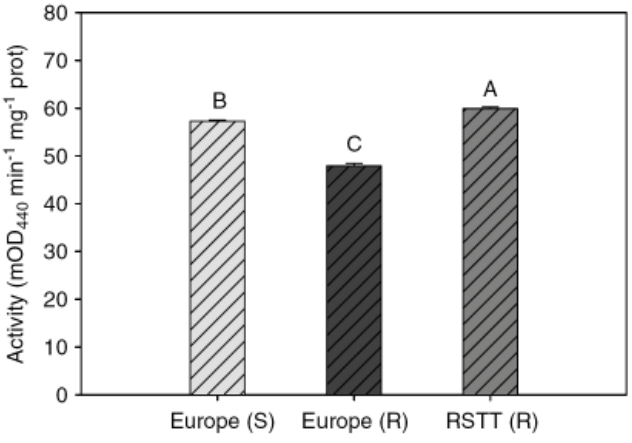

Figure 1. Total proteolytic activity from susceptible and Cry1Ab-selected colonies of *Ostrinia nubilalis*. Proteinase activity was measured with azocasein at pH 9.0. Each column represents the mean of three replicates from three samples ($P < 0.05$, LSD test; vertical bars, SE of the mean), and mOD_{440} = mili optical density at 440 nm wavelength.

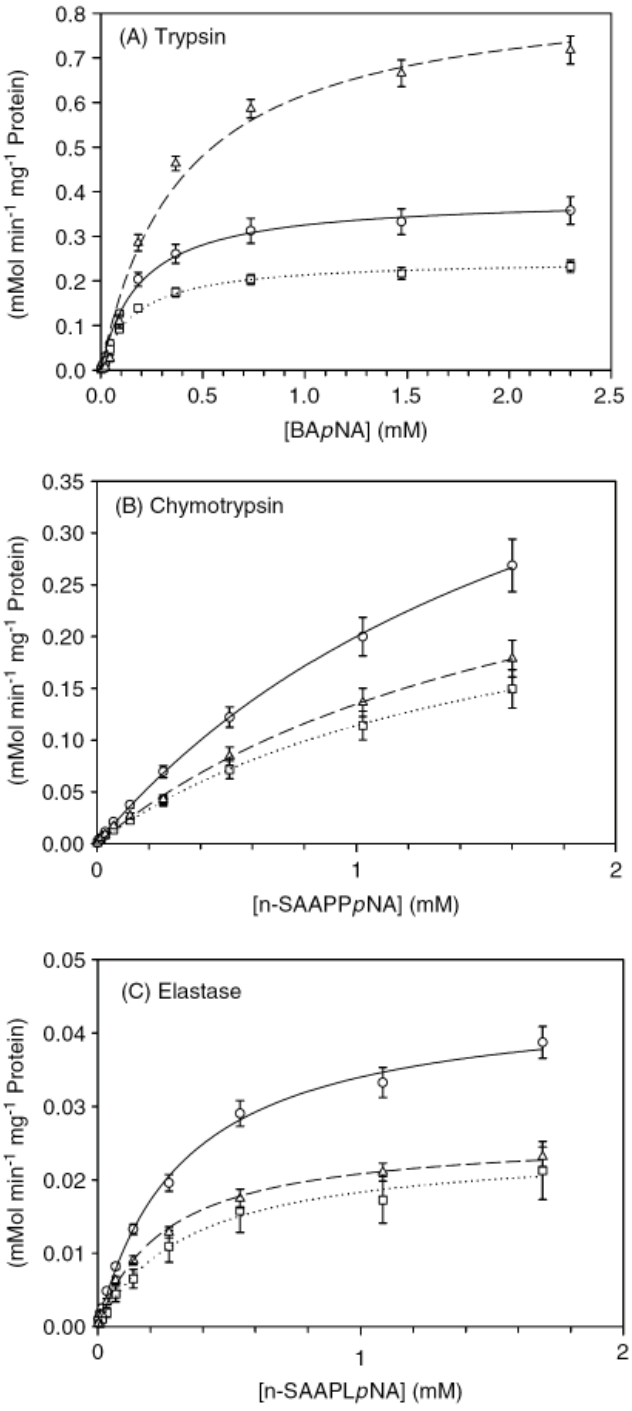

Figure 2. Specific activities of (A) trypsin-like, (B) chymotrypsin-like and (C) elastase-like proteinases from (○) Europe-S, (□) Europe-R, and (Δ) RSTT-R at 10 concentrations of substrate. Each curve represents an average of three different preparations (three replicates per sample). Bars represent the standard errors of the means (SEM). Non-linear regressions were significant at $P < 0.001$.

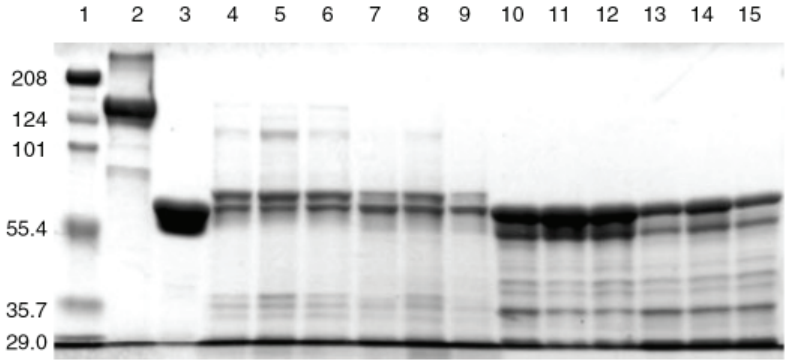

Figure 3. Cry1Ab protoxin and toxin forms incubated for 30 min and 1 h with luminal gut proteinases from a susceptible and two Cry1Ab-resistant colonies of *Ostrinia nubilalis*. Lane 1, marker; lanes 2 and 3, purified Cry1Ab protoxin and toxin, respectively; lanes 4-9, Cry1Ab protoxin incubations, 30 min (lanes 4-6) and 60 min (lanes 7-9); and lanes 10-15, Cry1Ab toxin incubations, 30 min (lanes 10-12) and 60 min (lanes 13-15), respectively. Lanes 4, 7, 10 and 13 represent the colony Europe-S. Lanes 5, 8, 11 and 14 represent the colony Europe-R. Lanes 6, 9, 12 and 15 represent the colony RSTT-R.

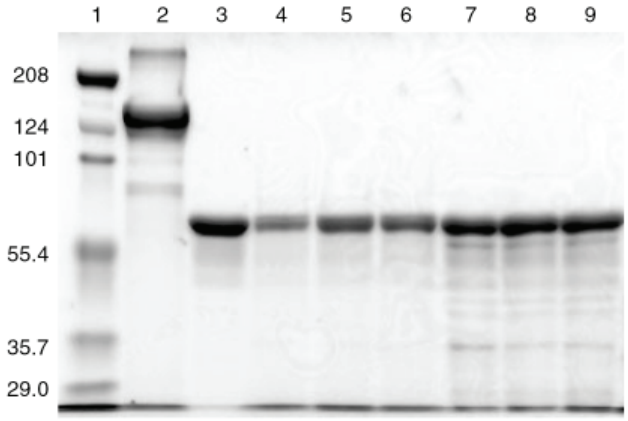

Figure 4. Cry1Ab toxin and protoxin incubated for 24 h with luminal gut proteinases from a susceptible and two Cry1Ab-resistant colonies of *Ostrinia nubilalis*. Lane 1, marker; lanes 2 and 3, purified Cry1Ab protoxin and Cry1Ab toxin, respectively; lanes 4-6, Cry1Ab protoxin incubations; and lanes 7-9, Cry1Ab toxin incubations. Lanes 4 and 7 represent the colony Europe-S. Lanes 5 and 8 represent the colony Europe-R. Lanes 6 and 9 represent the colony RSTT-R.

4 DISCUSSION AND CONCLUSIONS

Results of bioassays with both Cry1Ab protoxin and toxin indicated that levels of resistance in the selected colonies were significant higher for the activated toxin. Proteolytic processing of Cry proteins is an important step in toxin activation and potentially toxin degradation. These enzymes have been linked to both toxin potency[39,40] and to resistance.[15,21] Because of the observed differences in toxicity between full-length and truncated Cry1Ab among selected and control colonies, we examined changes in gut proteinases that might be related to resistance. Comparison of toxicity between Cry1Ab protoxin and toxin in *P interpunctella* indicated a higher level of resistance to the protoxin, and a reduction in protoxin activation was identified as the major resistance factor.[16]

In the present work, resistance was at least 18-fold higher to Cry1Ab toxin than to protoxin, suggesting that resistance involves factors other than changes in the rate of protoxin activation, and potentially multiple resistance mechanisms. Differences in proteolytic activities observed between resistant and susceptible *O. nubilalis* do not correlate with Cry1Ab protoxin susceptibility and do not seem to play a major role in the resistance to Cry1Ab toxin. A reduction of at least 36% in the activity of all proteinases tested was observed in Europe-R compared with the susceptible colony. Similarly, the RSTT-R colony exhibited a 42% reduction in the activities of chymotrypsin-like and elastase-like proteinases. In contrast, this colony exhibited at least 2-fold higher trypsin-like activity relative to the susceptible colony. The differences in activity of luminal gut proteinases among resistant and susceptible colonies are not consistent with *in vivo* Cry1Ab bioassays or with *in vitro* processing of Cry1Ab protoxin and toxin. Both protoxin and toxin seemed to be activated and degraded at similar rates by resistant and susceptible colonies, indicating proteinases do not play a major role in either resistance to Cry1Ab protoxin or to activated Cry1Ab.

Comparison of proteinase activities in luminal contents of susceptible and resistant populations have been reported in several insect species.[8,9,11,14,15,41,42] However, in most of these reports, insects were selected with multiple toxins from different *B. thuringiensis* strains, which might elicit a relatively non-specific mechanism of resistance such as proteinases-mediated activation.[8] In the present study, *O. nubilalis* colonies were selected with a single Cry1Ab protoxin, resulting in low levels of resistance to Cry1Ab protoxin, and it seems likely that the resistance is more related to changes in receptor binding. We reported previously[25] that resistance to Cry1Ab led to a high level of cross-resistance to Cry1Ac and a low level of cross-resistance to Cry1F. Because Cry1Ac and Cry1F have been reported to share high- and low-affinity receptors, respectively, with Cry1Ab,[43] *O. nubilalis* resistance to Cry1Ab seems more likely to be associated with reduced receptor affinity. In addition, lack of cross-resistance to Cry2Ab and Cry9C supports this hypothesis.[25]

Höfte and Whiteley[40] stated that the most obvious factors that may influence the host range of a crystal protein involve differences in the larval gut affecting the solubilization, processing efficiency, or both of the protoxin and the presence of specific toxin-binding sites (receptors) in the gut of different insects. Results of the present study suggest that resistance to Cry1Ab observed in Cry1Ab-selected *O. nubilalis* colonies is probably due to mechanisms other than proteolytic processing of toxin, such as binding site alteration or reduced pore formation.[22] Miranda *et al*[22] found that *Manduca sexta* (L) midgut extracts produced a more active toxin for pore formation assays than treatment with mammalian trypsin. In their report, the trypsinized toxin did not possess the first two cleavages at the *N*-terminus of Cry1Ab as observed with the insect midgut extract, and it seems probable that insect trypsins have different properties or specificity from their mammalian counterparts. Such differences between insect and mammalian proteinases might contribute to the observed differences in susceptibility between Cry1Ab protoxin and toxin among the selected and control colonies.

The involvement of gut proteinases in the resistance of *O. nubilalis* to Cry1Ab does not seem to be the major mechanism of resistance to Bt Cry1Ab in the selected colonies examined. Although differences in proteinase activities were apparent, such differences were not correlated with resistance and may be indicative of pleiotropic effects of the selection. Characterization of purified proteinases would clarify the differences found in the present work and help to explain their potential involvement in resistance to the Cry1Ab toxin. The results of this work indicate that multiple mechanisms of resistance are present in the selected colonies, and potentially target site alteration is the major mechanism occurring on them. Whether proteinases are involved in the resistance in these colonies, their role must be of minor importance.

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